ECOLOGY AND THERMAL INACTIVATION OF MICROBES
IN AND ON INTERPLANETARY SPACE VEHICLE
COMPONENTS

Twenty-fourth Quarterly Report of Progress

Research Project R-36-015-001

January 1, 1971 - March 31, 1971

Conducted by

Division of Microbiology - Cincinnati Research Laboratories
Bureau of Foods
Food and Drug Administration

Spacecraft Bioassay Laboratory
Cape Kennedy

CDC - Phoenix

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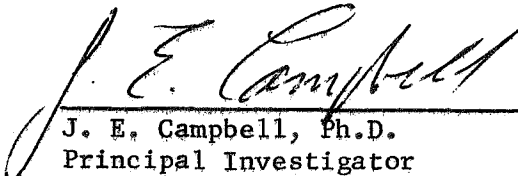
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EVALUATION OF A TERMINAL STERILIZATION PROCESS FOR UNMANNED LANDERS

Introduction

This project "Evaluation of a Terminal Sterilization Process for Unmanned Landers" was initiated at the request of the Planetary Quarantine Officer during the Spacecraft Sterilization Technology Seminar held in Atlanta, Georgia on April 15-16, 1970. The conduct of the work is being jointly undertaken by FDA, Cincinnati; CDC, Phoenix; and the Space Craft Bioassay Laboratory, K.S.C. The development of the plans and this progress report, however, actually represent the time and efforts of a large number of people including the staff of the Planetary Quarantine Office, the Planetary Quarantine Advisory Committee, consultants to this Committee, and contractors to the Office.

During the course of the work, it has become apparent that the objective of the project has two distinct and independent facets:

A. Development of an experimental system that is capable of detecting the acceptable levels of contamination.

B. Selection of an appropriate bioburden for the test system.

This report is concerned with Part A only.

I. OBJECTIVE

To determine in as direct a manner as possible if the sterilization cycle proposed for unmanned landers will meet the constraint that the contamination will be equal to or less than 1×10^{-3} organisms

(α = 0.01) when challenged with a bioburden consisting of the types and numbers of organisms expected to be found on the landers immediately after assembly.

II. A PLAN TO TEST TERMINAL STERILIZATION CYCLES FOR UNMANNED LANDERS

The purpose of a terminal sterilization cycle for unmanned landers is to insure, with some probability α, that no more than one spacecraft in one thousand will have terrestrial contamination. This original statement has been modified to mean that type of terrestrial contamination which is likely to survive on Mars. Some hypothetical conditions and growth restraints have been suggested by the Space Science Board of the NASNRC. The present study was designed to test terminal sterilization cycles on a laboratory scale.

This study is composed of two major elements that include a thermal source and units used to simulate the spacecraft or part of the spacecraft. Large numbers of units will be required for this study. For this reason, it was decided to simulate the heat cycle to be expected in the portion of the craft having the most thermal inertia. Stainless steel cups have been chosen for convenience in handling. Stainless steel strips or some other similar configuration would be just as good if they could be handled aseptically.

The sequence of tests is also a constraint in the experimentation since it is anticipated that the total number of experimental units will exceed 20,000. Each test takes a week including the incubation of samples. They could be set up so one unit is heated in each of n ovens. This is clearly too expensive. The tests can be run in one

oven in a sequential manner (i.e., one cup at a time). This has the advantage that tests on highly resistant bioburdens will end quickly. However, more than 6,000 tests will be needed to successfully examine one terminal sterilization cycle. It would take more than 100 years at one a week. Both of the above procedures were deemed impractical.

An alternative is to run large numbers of units in the oven at one time. The present capacity of the equipment developed for this study is 2,088. The main objection to this is that independence of cups is necessary in order for the statistical test to be valid. This assumption was satisfied by the two procedures noted above as each cup was to be heated independently of the others. However, the system composed of oven and cups can be used to determine the efficacy of heat cycles in short time. It will be necessary to test the independence assumption for this system.

The project is expected to progress through the following steps:

1. Testing of oven to program heating cycles.

2. Determine that sterile cups can be handled aseptically. Run sufficient samples to determine that the background contamination, if any, is less than 1×10^{-3}.

3. Test for independence of cups.

4. Test bioburden(s) with terminal sterilization cycle(s). Suggested numbers of observations to carry out 2, 3, and 4 above are given in the next section.

III. SUGGESTED OUTLINE FOR EVALUATION OF THERMAL STERILIZATION CYCLE

The system described above will produce results that are recorded as positive or negative. This occurs when the growth medium is poured into the cups after heating and growth or no growth is observed. If these binominal responses are independent, the following equation can be used to determine the number of negative results required.

$$e^{-\lambda n} = \alpha_1 \tag{1}$$

where α_1 = probability that n negative results would occur when the concentration is λ per cup

λ = concentration of organisms per cup

n = number of cups

This function is listed in Table 1 and plotted in Figure 1 for three values of α_1. If $\lambda = \alpha_1 = 0.001$, then n = 6908 negative cups must be observed. This means that there is only one chance in one thousand that the true concentration could be higher than $\lambda = 0.001$. An alternate problem is also of interest. Suppose x positive results are observed in n trials. Equation (2) shows the relation between concentration λ and α_1 level.

$$\binom{n}{x}\left(e^{-\lambda}\right)^{n-x}\left(1-e^{-\lambda}\right)^{x} = \alpha_1 \tag{2}$$

This requires a trial and error solution. However, if α_1 and n are fixed, the number of possible solutions is reduced.

With the use of (1) and (2) above, it now is possible to examine a test sequence for terminal heating cycles. An outline of suggested

steps is given in Figure 2. Three divisions of the experiment are considered. First, it is desired to examine the technique with sterile cups to insure that background contamination is below the $\lambda = 0.001$ level required in the final test. The next step is to test for independence of cups before the heating cycles are examined. Plans can be constructed to allow for decisions at several stages of the experiment.

At this point the number of possible choices for variables should be considered since they affect the solution to the problem. Some of these variables are listed below:

1. Type of spore or spores used as a bioburden.

2. Initial load of bioburden (i.e., 1×10^6 or organisms per cup).

3. Heating cycle.

    a. Temperature (125°C).

    b. Corrected time of heating at temperature (t_p).

4. Desired endpoint concentration (1×10^{-3}).

5. Probability level for confidence limits (α).

The rationale for 1 and 2 are not in the province of the statistician but have a marked effect on the result. The choice of spore(s) depends on what is most likely to be on the spacecraft and the initial load also depends on the expected spacecraft load. At present, the temperature for the heating cycle is set at 125°C but the time at temperature (t_p) is subject to change. The same is true for the probability level of the confidence limits (α) which is not fixed. A value of $\alpha = 0.05$,

0.01, and 0.001 was used to compare the magnitude of numbers required in the study. The endpoint concentration will be assumed as one organism per thousand units.

In Figure 2 it is first necessary to perform a test on sterile cups to determine if asceptic technique is used. As stated above, the endpoint is 1×10^{-3}. If the α level is 0.05, 0.01, or 0.001, then n equal 2996, 4606, or 6908 cups would be needed, with all results reported negative, in order to verify that the background is not too high to interfere with the studies. The results for $\alpha = 0.01$ are suggested for this study. If either 0 or 1 negatives is allowed, then n must be 6630 at the $\alpha = 0.01$ level. Since only 2088 cups can be tested in an oven at once, the procedure must be repeated four times.

The second step in the test sequence (Figure 2) is to examine the independence assumption. This can be accomplished in several ways. One of the procedures is given below. It is necessary to perform a preliminary test on the oven using a spore of known thermal resistance and Bacillus subtilis can be used for this purpose. All cups are to be inoculated with these spores and held for time, $t_{1/2}$, when one-half of all cups are negative. MPN estimates from each of the 12 rows in the oven are used to determine if gross differences occur within various parts of the oven. If the results appear uniform, then 100 cups per shelf are to be randomly chosen and inoculated. These 300 cups and 300 controls are to be heated time $t_{1/2}$ and processed so as to obtain a plate count per cup. The distribution of the counts is then to be

examined by an χ^2 goodness of fit test. If this test is not signifi-cant, then we will continue to assume that the independence assumption is valid. At this point the heating cycles can be examined.

It is expected that several choices of bioburden at differing initial loadings will be tested. No doubt more than one t_p will be suggested. However, the number of units required to test the end-point, $\lambda = 0.001$, with confidence level $\alpha = 0.01$ will still be 4606. If all 4606 cups are negative for a given initial loading of a bio-burden heated for a t_p, then the cycle will be deemed useful. An estimate of λ at level $\alpha = 0.01$ can be obtained when positive cups are observed. Even if the heating cycle is not sufficient, an esti-mate of its relative merits can still be noted.

The 4606 cups would be run in five batches to allow half the cups in each run to serve as controls. Thus, a process can be evalu-ated as follows:

    a. It meets the required λ standard.

    b. It does not meet the standard but an estimate of λ

       for the same α used in the test is presented.

It is not necessary to assume that any of the spores follows the log linear model. The test is to determine if sufficient inactivation has taken place at time t_p so that there is only one chance in one thousand that the concentration is $\lambda = 0.001$. It is implied that the spores cannot be reactivated once they reach Mars.

IV. CURRENT STATUS OF EQUIPMENT AND EXPERIMENTAL METHODS

Equipment necessary for the evaluation of the terminal steriliza-tion cycles was designed, fabricated, and assembled in Cincinnati during

the fall of 1970. After preliminary tests, the equipment was delivered in January 1971 to the Spacecraft Bioassay Laboratory for further testing and use.

The general arrangement of the test assembly is shown in Figure 3. The oven is a commercially available high temperature vacuum oven which has been equipped with the following accessories:

    a. Temperature programmer

    b. Temperature sensors

    c. Gas flow controller

    d. Gas flow monitor

    e. Moisture monitor

The test fixtures (simulated spacecrafts) are small stainless steel cups identical to those used by the Cincinnati Laboratory in its project on "The Ecology and Thermal Inactivation of Microbes In and On Interplanetary Space Vehicle Components." The configuration of the oven allows for the simultaneous exposure of 2,088 cups.

During November 1970 an employee from the spacecraft bioassay laboratory was temporarily assigned to Cincinnati for about a week to learn the techniques for handling the fixtures. The training included practice in the procedure for rapid inoculation of the cups with microorganisms, accounting for individual cups during all phases of handling, culturing survivors after heat treatment and in scoring cups for survivors. With only slight modification, these techniques appear to be applicable to the present study.

V. PRELIMINARY EVALUATION OF SYSTEM

In all of the work reported below, "dry" conditions have been maintained during the heating and cooling cycle by passing dry nitrogen into the oven at a rate to allow complete change of gas approximately every 15 minutes. Commercially available dry nitrogen (MIL-P-27401B) has been found satisfactory without further treatment in that the moisture level measured at sterilizing temperatures is less than 0.001 micrograms per ml. Temperature measurements of various parts of the oven were found to vary about 1/2°C. The temperature variation during cycling of the oven (on and off periods) is approximately the same. The first series of experiments was related to the integrity of the oven and the biobarrier.

The oven was filled with 2,088 empty cups, sealed, the temperature raised to 150°C for a minimum of 6 hours, and allowed to cool to room temperature. The samples of the cups were then removed, nutrient media added to each cup, and allowed to incubate for 7 days. The cups were then inspected and scored for growth. Thus far, three replicate experiments have been conducted. In the first and second experiment, no positives were observed. In the third experiment, two positives were observed. These data were pooled and subjected to an appropriate statistical analysis. The estimated level of contamination was found to be 0.003 or 3 contaminated cups in 10,000. Evaluation in terms of the constraint is not met, however, because there are 4 chances in 100 that the background contamination will exceed 0.001, or 1 contaminated cup per 1000. Additional experiments are being carried out,

with modifications in technique, to determine if the necessary
constraint can be realized.

In the test for independence, the oven was loaded with cups
each containing 1×10^6 B. subtilis var. niger. The oven was run
through the heating cycle shown in Figure 4 except at a rate four
times as fast as shown, which provided the equivalent of approximately
3-1/2 hours at 125°C. The samples were removed and media placed in
each cup. They were then incubated for a period of 7 days and scored
as shown in Figure 5. Each number recorded represents the number of
positive samples observed in a tray containing 29 cups. The small
differences in the estimated MPN between rows and shelves were found
to be significant and, accordingly, not in keeping with the constraint
required in the plan. Several changes are being made in the oven re-
lating to the inlet and outlet of gas, the method of creating turbulence
in the oven and in modification of the heating cycle, in order to de-
termine if these constraints can be met.

Table 1

Number of Negative Observations Required to Determine

That Concentration λ Will Occur With Probability α

Concentration of organisms per cup λ	α		
	0.05	0.01	0.001
0.1	30	46	69
0.01	300	461	691
0.001	2,996	4,606	6,908
0.0001	29,958	46,052	69,078

λ.—ORGANISMS PER CUP

n—SAMPLE SIZE (All Tubes Negative)
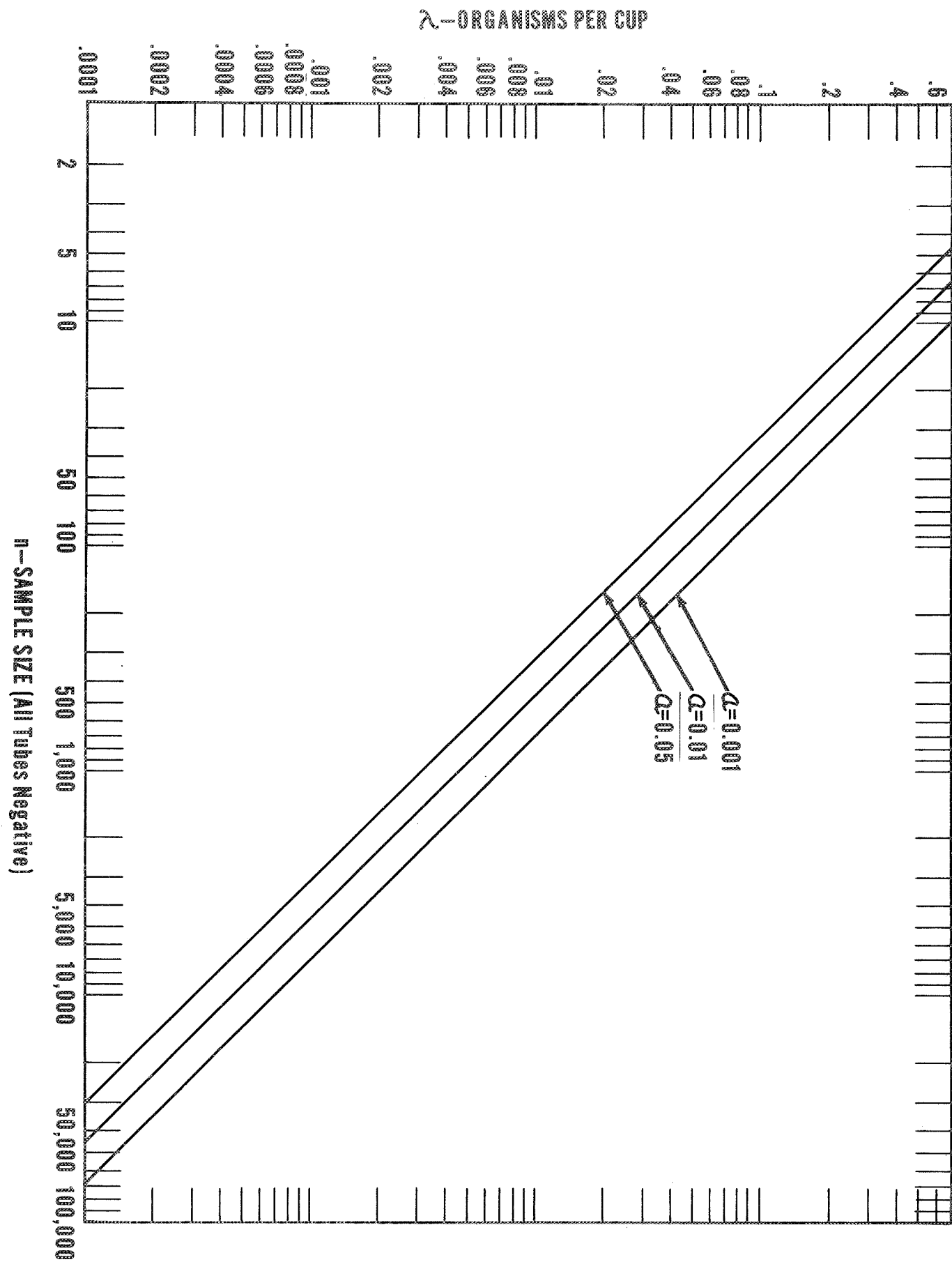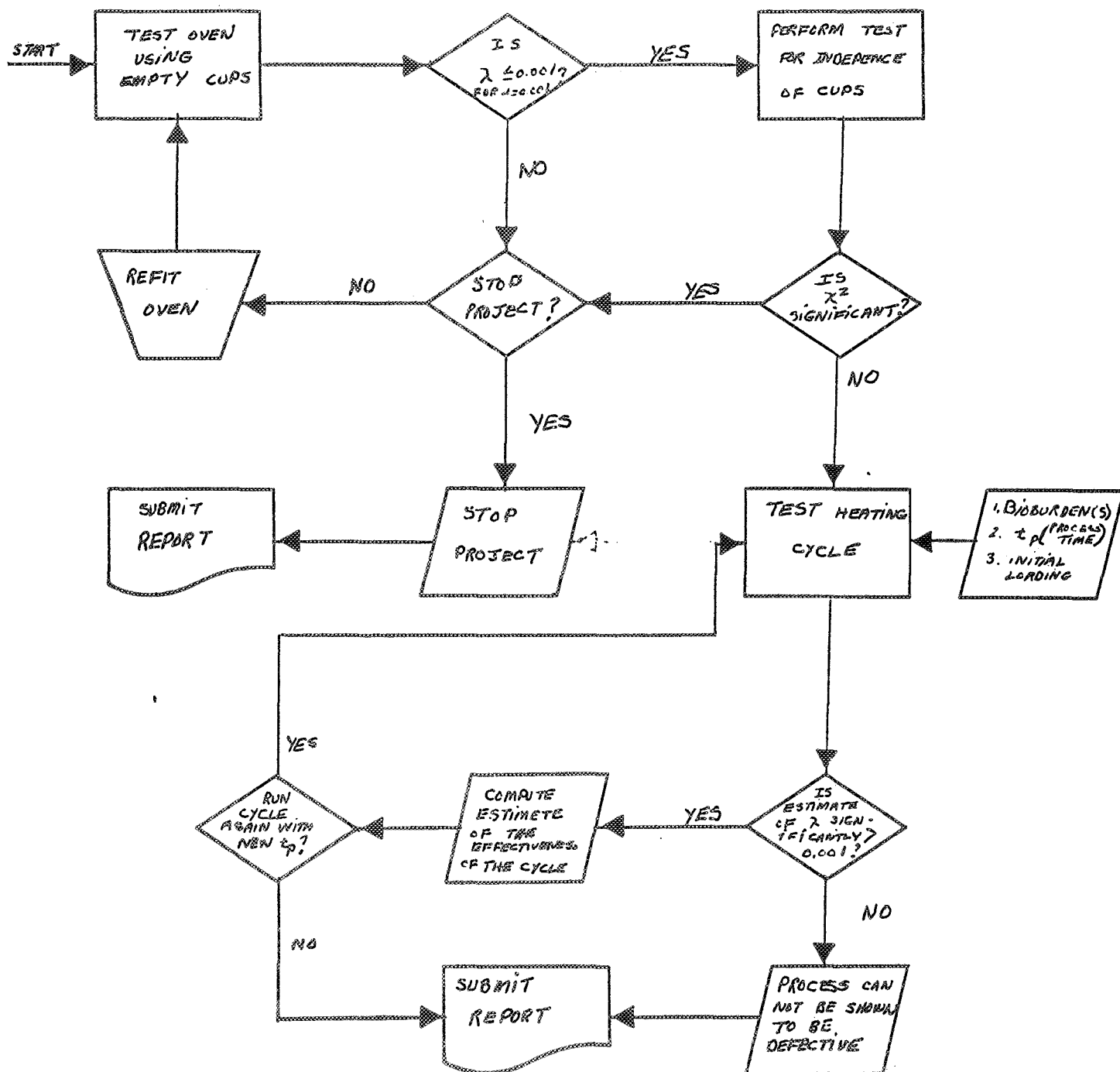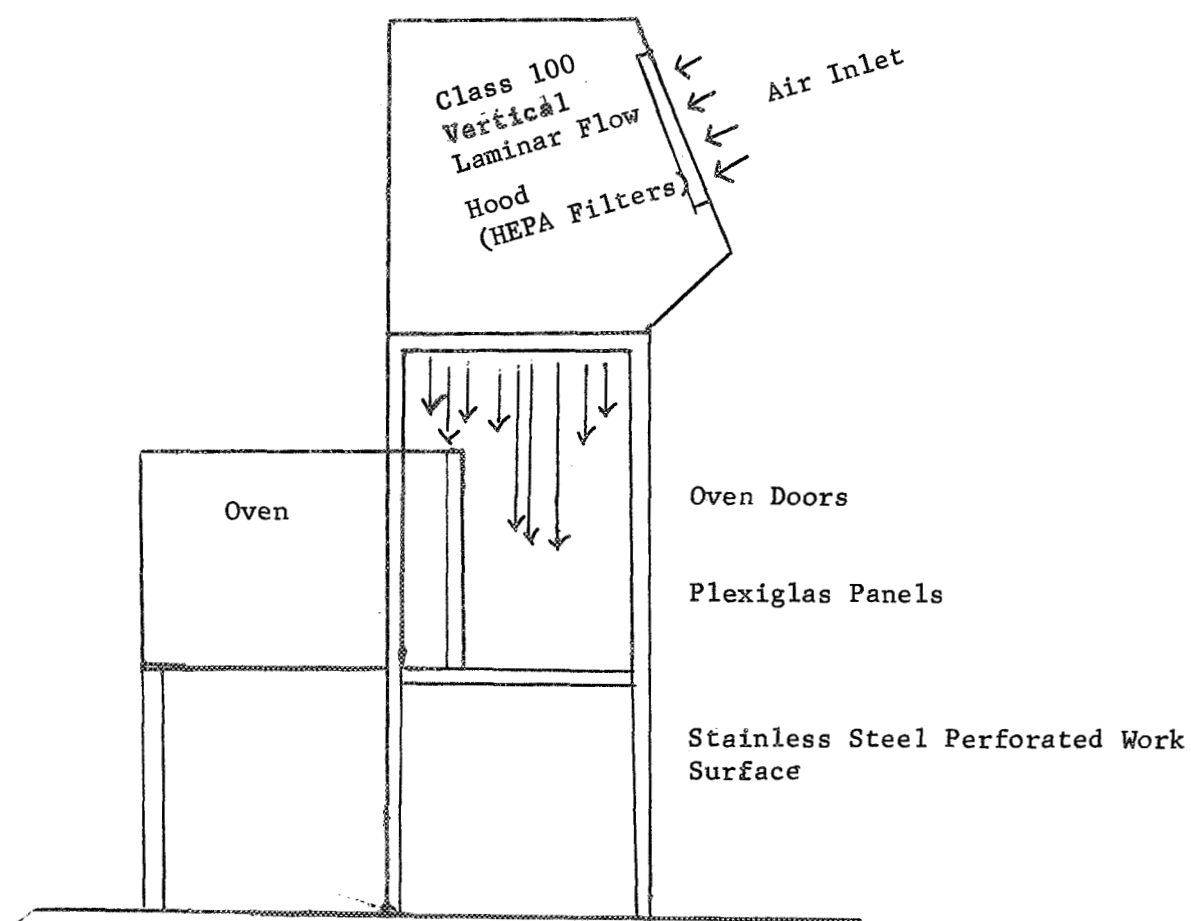
α=0.05
α=0.01
α=0.001

Figure 1

Figure 2. Suggested work plan.

Figure 3. General arrangement of biobarrier and oven

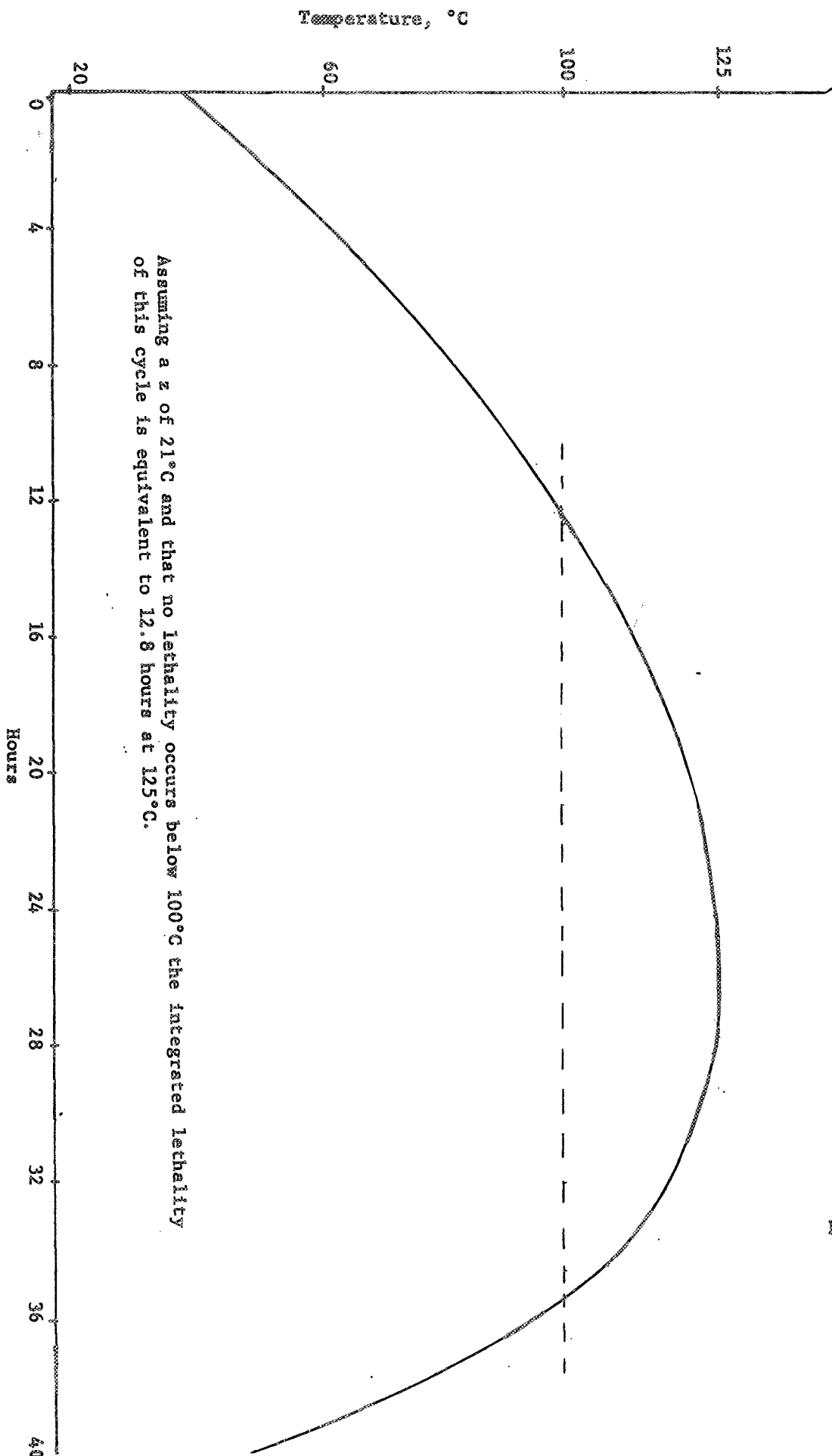Figure 4. Proposed terminal sterilization cycle temperature-time profile.

Assuming a z of 21°C and that no lethality occurs below 100°C the integrated lethality of this cycle is equivalent to 12.8 hours at 125°C.

Figure 4

Figure 5

Test for Independence

Number of Positive Cups per Tray*

0	0	0	0
2	1	0	0
0	2	0	2
0	1	2	0
0	1	0	5
5	2	4	3

Shelf #3

Total	7	7	6	10	30
MPN	0.041	0.041	0.035	0.059	

0	4	0	0
8	0	0	0
1	5	0	3
2	0	0	0
2	0	2	0
2	-	-	1

Shelf #2

Total	15	9	2	4	30
MPN	0.090	0.064	0.014	0.028	

0	1	4	3
0	0	3	3
0	3	1	0
0	3	2	4
6	7	1	0
2	5	2	0

Shelf #1

Total	8	19	13	10	50
MPN	0.047	0.116	0.078	0.059	

$\overline{110}$ (2030)

* Each tray contains 29 cups & a marker.